Distribution of a Wound Epithelium Antigen in Embryonic Tissues of Newts and Salamanders[1]

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ABSTRACT. Monoclonal antibody WE3 (mAb WE3) reacts to the majority of cells of the wound epithelium during limb regeneration, but to only a small minority of cells of skin epidermis. Since both the apical ectoderm of the limb bud and the wound epithelium of regenerating limbs are important to limb outgrowth, it was of interest to determine whether the WE3 antigen was shared by these two developmentally important tissues. To help determine the significance of WE3 reactive cells in the wound epithelium and other tissues of the adult, we investigated whether mAb WE3 reacted to non-limb tissue in embryos of newts (Notophthalmus) and Ambystoma. Ab tests were performed at various stages of development; reactivity of mAb WE3 was similar in embryos of three species. Ectoderm cells of the limb bud did not react to WE3, but occasional reactivity was observed in gland-like structures in the anterior body ectoderm. Reactivity appeared in the early developing pronephros, foregut, heart, dorsal aorta, and notochord, beginning at late tail-bud stages. These results show that some cell types exhibit WE3 reactivity in both the adult and embryo. Other cell types which react to WE3 in the adult are not reactive during embryonic stages.

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INTRODUCTION

Several monoclonal antibodies (mAbs) have been obtained which react to antigens of regenerating adult newt forelimbs (Kintner and Brockes 1984, Tassava et al. 1986, Goldhamer and Tassava 1986, Tassava et al. 1987). One of these, mAb WE3, reacts to the vast majority of cells of the wound epithelium during adult newt limb regeneration but to only a few cells of the skin epidermis (Tassava et al. 1986). Reactivity of mAb WE3 first appears in the wound epithelium during the second week post-amputation, remains high during blastema stages, and by late digit stages decreases to the level seen in skin epidermis (Tassava et al. 1986, 1987). A comprehensive tissue survey (Goldhamer, Tomlinson, and Tassava, unpubl. data) revealed that WE3 reactivity is present in many secretory/transport cell types, including cells of the kidney, gastrointestinal tract, endothelium of blood vessels, and perineurium of nerves. The goal of the present study was to examine the ontogenetic appearance of WE3 reactivity in embryos of newts, axolotls, and local *Ambystoma*. Because mAb WE3 reactivity in the wound epithelium correlates with the period of most active regenerative outgrowth, it was particularly important to determine whether WE3 appeared in the limb bud ectoderm during embryonic limb outgrowth.

MATERIALS AND METHODS

Axolotl (*Ambystoma mexicanum*) embryos were from a laboratory spawning of adults raised from larvae obtained from the Indiana University axolotl colony. Spotted salamander (*Ambystoma maculatum*) embryos were collected at blastula stages from local ponds in mid-March. Adult newts (*Notophthalmus*) in breeding condition (males had claspers and nuptial pads; females were gravid; amplexus behavior was apparent; Gage 1891) were brought into the laboratory and used immediately or maintained for 1 to 3 weeks in aerated tap water in a refrigerator at 6°C. Muscle (control tissue) or pituitary glands were dissected from both males and females, shredded with watchmaker's forceps in 0.2 mL of Holtfreter's solution, drawn into a 1-mL tuberculin syringe, and the total 0.2 mL of suspension was forced through a 25-gauge needle during intraperitoneal injection into each female. Each female newt was then placed singly into a 15-cm diameter plastic dish containing 500 mL of aerated tap water and two branches of *Elodea*, each with several dozen leaves. After injection, newts were maintained at 24°C under the combined conditions of room and natural (windows) lighting. At daily intervals, leaves were inspected for eggs. Usually, a single egg was wrapped in a single fold of a single leaf. Occasionally, two eggs were wrapped in a single leaf.

Staging of newt, axolotl and spotted salamander embryos was according to Harrison's table (Hamburger 1960) for embryos of *A. maculatum*. At the desired stages, beginning at early gastrulation and continuing through hatching, embryos were removed from jelly coats, frozen in OCT compound (Tissue Tec; Fisher) in a bath of isopropyl alcohol and dry ice, and sectioned transversely at 10 μ in a cryostat. Sections were air-dried before being incubated with antibodies.

Monoclonal antibody WE3 was obtained as previously described (Tassava et al. 1986) with sonicated mid- and late-bud blastemas as immunogen. Sections were incubated with mAb WE3 (primary Ab; 50× dilution of ammonium sulfate precipitated Ab) for 1 h in a humid atmosphere, then washed three times in phosphate-buffered saline (PBS) with 0.05% Triton X-100 and once in PBS without Triton. Rhodamine-labeled goat antimouse IgG (secondary Ab; Cappel; 50× dilution in PBS) was then applied for 1 h. Slides were washed as above, mounted with glycerol: PBS (95:5), and viewed by indirect immunofluorescence. Controls included other mAbs, secondary antibody alone, or sections without primary or secondary antibody.

In order to insure identification of embryonic tissues and organs, various stages of embryos of all three species were fixed in Bouin's fluid, prepared for paraffin histology, sectioned transversely at 10 μ, and stained with hematoxylin and eosin (H&E). In other cases, after examination of antibody-reacted sections by indirect immunofluorescence, cover slips were removed and sections were stained with H&E and examined by light microscopy.

RESULTS AND DISCUSSION

INDUCTION OF SPAWNING. Under laboratory conditions, female newts not given injections of pituitary gland homogenates occasionally released eggs but generally these were infertile. In preliminary experiments we observed fertile spawnings after injections of two, three, five, or eight pituitary glands (injected as homogenates), but the lesser numbers of glands were as effective as the greater numbers. Egg numbers spawned by each female ranged from 2 to 40. Fertile spawnings were not obtained from newts stored in the laboratory for over 2 weeks. It was not necessary to pair males with females; when collected during the breeding sea-

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son, most female newts already have acquired spermatophores (Adams 1930, Kaylor 1937).

REACTIVITY TO MONOCLONAL AB WE3. Cryostat sections of newt and *Ambystoma* embryos were tested for reactivity to mAb WE3 at stages from gastrulation through late tail bud. It was of particular interest to examine the developing notochord, beginning at gastrulation, since preliminary studies showed WE3 reactivity in this tissue (Tassava and Acton unpubl. data, Neff and Tassava unpubl. data). For purposes of comparison, newts and axolotls were staged as closely as possible to stages of *A. maculatum,* according to Harrison's table (Hamburger 1960, Sturdee and Connock 1975). From Table 1, it can be seen that reactivity to WE3 was not present in the chordamesoderm at gastrulation (stages 10-12) nor in the early developing notochord through neurulation (stages 13-24), and early tailbud (stages 30). However, weak reactivity appeared in the notochord at stages 35 and 36 (early limb bud) and became strong by stage 40 (Fig. 1). It is during this time that the notochord undergoes a vacuolization or "expansion" process (Takaya 1973, Malacinski and Youn 1982). The notochord cells enlarge and expand further as larger vacuoles are formed in the cytoplasm. It is of interest that WE3 reactivity is present just inside the periphery of the cells, not throughout the vacuolated cytoplasm (also confirmed with hematoxylin-eosin staining, data not shown). Other data from our laboratory suggest that WE3 reactivity is associated with secretory or transport cell types (Goldhamer, Tomlinson, Tassava unpubl. data). Therefore, WE3 reactivity in the notochord may be associated with uptake of materials involved in vacuole formation. Which, if any, of the many presumed functions of the notochord (Malacinski and Youn 1982) might be related to this vacuolization process is not clear. WE3 reactivity persists in the notochord into larval stages (Tassava unpubl. data), so WE3 reactivity is possibly associated with some other aspect of notochord remodeling or function.

WE3 reactivity also was present in the gut endoderm, first in the early developing foregut and later in the hindgut, and in the pronephros, dorsal aorta and heart primordium (Figs. 1-3; Table 1). These latter cell types initiated and then exhibited increased reactivity during the same time period (stages 36-40) as did the notochord. Cells of the gut, kidney, and vascular endothelium also reacted to WE3 in the adult. We cannot at present assign a functional role for the embryonic cells which are WE3 positive. It can be hypothesized, as discussed above, that WE3 reactive cells of embryos are already exhibiting or will later assume a transport or secretory function (Goldhamer, Tomlinson, and Tassava unpubl. data).

The limb bud mesenchyme, developing brain and spinal cord, cornea, conjunctiva, and retina were all negative through stage 40 (Table 1). Recently we have observed (Goldhamer, Tomlinson, and Tassava unpubl. data) that the conjunctiva (but not cornea) and the ependymal cells of the spinal cord are WE3 reactive in the adult newt. Therefore, these tissues apparently acquire their reactivity later in development, during larval stages, or at metamorphosis; studies are underway to further examine the timing of WE3 appearance during the newt life cycle.

In the ectoderm, an occasional isolated cell and sometimes small groups of cells, resembling glands, were WE3 reactive, particularly in the anterior end (not shown); dermal glands were not yet present. A more detailed study of ectoderm reactivity throughout embryonic development is underway. Important to the present study was the observation that the ectoderm cells of the limb bud were not reactive to mAb WE3 (Fig. 4). During adult newt limb regeneration, at bud stages, most of the cells of the wound epithelium are WE3 reactive (Tassava et al. 1986, 1987). Clearly, the ectoderm of the limb bud does not exhibit comparable reactivity.

The wound epithelium of regenerating limbs may have different functions and therefore different developmental antigens than the limb bud ectoderm. Perhaps, as appears to be true for the 22/18 antigen (Fekete and Brockes 1987), WE3 appearance during regeneration is somehow related to injury phenomena. In this regard, it is of interest that WE3 reactivity is absent in the developing retina of the embryo (Table 1) but is present in the regenerating retina of the adult (Tassava and Mitashov unpubl. data). There is also the possibility that functionally related isoforms are present in limb bud ectoderm and developing retina that do not have the antigenic determinant recognized by mAb WE3.

TABLE 1

Stage and tissue specificity of WE3 reactivity in embryos of newts, axolotls, and local Ambystoma.

	Embryonic Stage When WE3 Reactive				
Tissue	10-12 Gastrula	13-25 Neurula	30 Tail-bud	35 E. Limb-bud*	40 M. Limb-bud*
Body ectoderm	−	−	−	+	+
Limb ectoderm	−	−	−	−	−
Limb mesenchyme	−	−	−	−	−
Notocord	−	−	−	+	+
Foregut	−	−	−	+	+
Hindgut	−	−	−	+	+
Dorsal aorta	−	−	−	+	+
Heart primordium	−	−	−	+	+
Pronephros	−	−	−	+	+
Spinal cord	−	−	−	−	−
Retina	−	−	−	−	−
Conjunctiva	−	−	−	−	−

*E. = Early; M. = Middle.

FIGURE 1. A montage of two fluorescence micrographs illustrating WE3 reactivity within the notocord (nc) and in the hind gut endoderm (hg) of the newt embryo at stage 36. Only an occasional ectoderm cell is WE3+ (arrow). Similar reactivity to WE3 was seen at comparable stages in embryos of axolotls and spotted salamanders. Autofluorescence, which is not above background, is seen in the developing mesoderm (m) masses. Note that the spinal cord (sc) is not reactive to WE3. ×70. Rhodamine 2nd antibody.

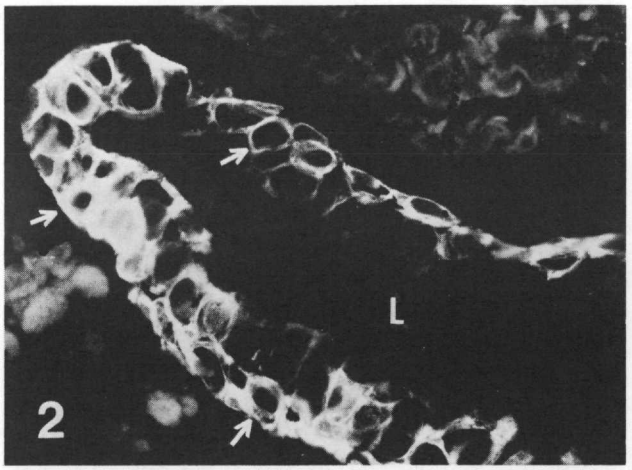

FIGURE 2. A fluorescence micrograph illustrating WE3 reactivity in the lateral area of the foregut (pharynx) cells (arrows) of a newt embryo at stage 34. Note that as in the adult (Tassava et al. 1986) the antigen is intracellular. Similar reactivity to WE3 was seen at comparable stages in embryos of axolotls and spotted salamanders. L = lumen. The mesenchyme lateral and dorsal to the gut exhibits autofluorescence which is not above background. ×300. Rhodamine 2nd antibody.

FIGURE 3. A fluorescence micrograph illustrating WE3 reactivity within the pronephros (arrows) of the newt embryo at stage 35. The most reactive cells are those lining the lumen (L). Similar reactivity was seen at comparable stages in embryos of axolotls and spotted salamanders. ×300. Rhodamine 2nd antibody.

Differences between adult and embryo in reactivity to mAb 22/18 have been described by Fekete and Brockes (1987). Whereas mAb 22/18 reacts to a subset of mesenchymal cells of the regenerating adult newt limb blastema (Kintner and Brockes 1984), reactivity is generally absent in other cell types of the body. In contrast, during embryonic development, reactivity of 22/18 is largely absent in the limb bud mesenchyme but is present in the body ectoderm, a restricted area of the aorta, glial cells of the neural tube, and lens (Fekete and Brockes 1987). Examining the ontogenetic appearance of reactivity of other mAbs to which regenerating limb components are reactive (Goldhamer and Tassava 1986) will ultimately lead to identification of common and unique antigens to these systems.

The present results show that mAb WE3 is not species-specific in that reactivity to *Ambystoma* was seen; tests of WE3 reactivity to axolotl and *A. maculatum* wound epithelium can now be carried out at larval and adult stages. In contrast, mAb 22/18 is somewhat species-specific in that it will react to *Notophthalmus* and *Pleurodeles* but not to *Ambystoma* (Fekete and Brockes 1987).

The significance of the WE3 antigen in the wound epithelium during adult newt limb regeneration and in other reactive body cells is not yet clear. Additional studies, designed to extract and characterize the WE3 antigen, may provide results important to correlating this antigen with a specific cellular function and/or structure.

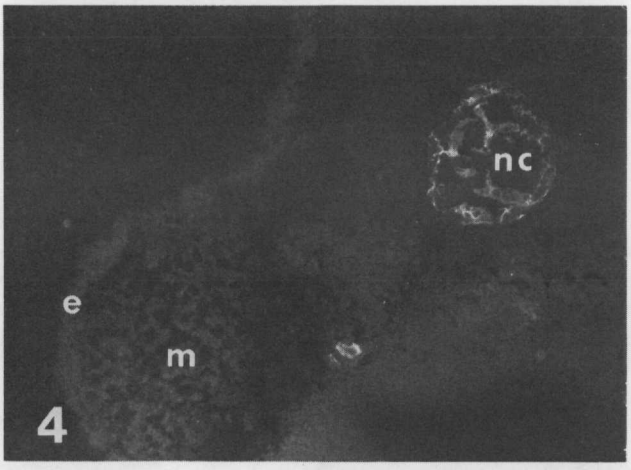

FIGURE 4. A fluorescence micrograph illustrating absence of WE3 reactivity within cells of the newt embryo limb bud ectoderm (e) and mesenchyme (m) at stage 35. The notocord (nc) exhibits WE3 reactivity. The spot of reactivity just medial to the limb bud is the anterior end of the pronephros. ×60. Rhodamine 2nd antibody.

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